Research Article

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Toxoplasma gondii RPL40 is a circulating antigen with immune protection effect

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Abstract: Screening and identification of protective antigens are essential for the prevention of infections with *Toxoplasma gondii* (Nicolle et Manceaux, 1908). In our previous study, *T. gondii* ribosomal-ubiquitin protein L40 (TgRPL40) was identified as a circulating antigen. However, the function and protective value of TgRPL40 was unknown. In the current study, recombinant TgRPL40 was expressed in *Escherichia coli* BL21 and antibody was prepared. Western blotting analysis indicated that TgRPL40 was present in circulating antigens and excretory/secretory antigens (ESA). Immunofluorescence and immunoelectron microscopy analysis revealed that TgRPL40 protein is widely distributed in the tachyzoites. Immunisation with recombinant TgRPL40 prolonged the survival of mice infected with tachyzoites. Quantitative real-time polymerase chain reaction analysis showed that immunisation with recombinant TgRPL40 reduced the parasite burden in blood, liver, spleen and brain of mice infected with tachyzoites. These observations indicate that TgRPL40 is a circulating antigen and is an effector of immune protection against acute *T. gondii* infection.

Keywords: protein expression, subcellular localisation, protective effect

Toxoplasma gondii (Nicolle et Manceaux, 1908) is a major threat to human health and animal production. Infection with *T. gondii* is difficult to prevent and can cause serious illness. The parasite, which can infect all warm-blooded animals and proliferate in almost all nucleated cells, is the most widespread parasitic protist (Hunter and Sibley 2012). The majority of animals infected with *T. gondii*, including humans, typically exhibit no obvious clinical symptoms. However, serious and even life-threatening clinical symptoms can be present in organ transplant and immunosuppressed patients at the time of the first infection with *T. gondii* Robert-Gangneux and Darde (2012). Additionally, infection with *T. gondii* during early pregnancy can lead to abortion and fetal malformation (Hill and Dubey 2013, Mahmoudvand et al. 2015). Given the lack of effective drug treatments for *T. gondii* infection, the development of preventive strategies are of particular importance (Elmore et al. 2010, Zhou et al. 2016).

Immunisation with virulence factor-based vaccines is an effective strategy for the prevention of toxoplasmosis (Cong et al. 2011, Yin et al. 2015). Previous studies have shown that immunisation with *T. gondii* recombinant proteins, including cyclophilin, calcium-dependent protein kinase 1, and actin, can stimulate the production of specific anti-

bodies with immune protective effects (Huang et al. 2012, Yu et al. 2013, Chen et al. 2014). A recent study also indicated that circulating antigens (CAG) play indispensable roles in the pathological processes of *T. gondii* infection (Xue et al. 2016). Excretory/secretory antigens (ESA) are a general term for a class of proteins secreted by *T. gondii* in serum-free medium *in vitro*. CAG is the protein components of *T. gondii* in the blood of the host suffering acute toxoplasmosis. Previous studies have shown that most of the components of ESA and CAG are the same, such as surface antigens (SAG), micronemal proteins (MIC), dense granular proteins (GRA), and rhoptry proteins (ROP). (Zhou et al. 2005, Mattos et al. 2011, Meira et al. 2011).

Immunisation with ESA, which represent the majority of CAG, can effectively prolong the survival of mice infected with *T. gondii* Costa-Silva et al. (2008). Immunisation with recombinant ESA proteins, including SAG1, SAG2, ROP2, and GRA1, can also provide protective effects (Machado et al. 2010, Khosroshahi et al. 2012, Wu et al. 2012). However, antibodies targeting one or several virulence factors do not provide sufficient immune protective effects. In order to develop more effective protective strategies, more virulence factors with greater immunogenicity need to be further identified.

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Ribosomal-ubiquitin protein L40 (RPL40) is fusion expressed with ubiquitin in eukaryotic cells. The fusion protein (Ub_{L40}) is a component of ribosome. Ub_{L40} can be decomposed into a ubiquitin monomer and a RPL40 molecule catalysed by a specific protease (Khatun et al. 2013). RPL40 regulates p53 expression in ribosomal protein – murine double minute 2 – p53 pathway (Zhou et al. 2019). In our previous study, we identified *T. gondii* ribosomal-ubiquitin protein L40 (TgRPL40) as a CAg component in the sera of dogs acutely infected with *T. gondii* Xue et al. (2016).

The results also suggested that TgRPL40 might be an essential virulence factor and may play a role in the pathogenesis of acute infection with *T. gondii* Xue et al. (2016). The aim of the current study was to investigate the localisation of TgRPL40 in tachyzoites. Considering that TgRPL40 can stimulate the body to produce antibodies, we evaluated the protective effect of immunising the virulence factor on *T. gondii* acute infection by immunising mice with recombinant TgRPL40.

MATERIALS AND METHODS

Toxoplasma gondii, cells, and animals

Toxoplasma gondii RH parasites and Vero cells were stored at the Parasite Laboratory of the Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Science (Shanghai, China). Vero cells were propagated at 37°C and 5% CO₂ in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 20 mg/ml penicillin and 20 mg/ml streptomycin. *Toxoplasma gondii* was grown and maintained in Vero cells.

Female ICR mice (body weight ~25 g each) were purchased from the Shanghai Laboratory Animal Center, Chinese Academy of Science (Shanghai, China). All of the animals in the experiments were raised at the Animal Laboratory Center of the Shanghai Veterinary Research Institute in a sterile room and were fed sterilised food and water. The study was approved by the Animal Care and Use Committee of the Shanghai Veterinary Research Institute. Animals were handled in strict accordance with the animal protection law of the People's Republic of China (released on 18/09/2009) and the National Standards for Laboratory Animals in China (released on 01/05/2002).

Bioinformatics analysis

The secondary structure of TgRPL40 (GenBank: XP_002368400.1) was analysed using Protean software (DNASTar, Madison, WI, USA). The protein sequence of TgRPL40 was analysed using NCBI BLAST online tools (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). MegAlign software (DNASTar) was used for evolutionary tree analysis of the TgRPL40 gene sequence. Similarity comparisons with previously reported gene sequences were conducted using the online BLAST tool and DNAMAN 3.0 (Lynnon Biosoft, San Ramon, CA, USA). Multiple sequence alignment and phylogenetic analyses were conducted on the basis of observed similarities. Sequences were aligned with ClustalW2 (<https://www.ebi.ac.uk/Tools/msa/clustalw2/>). The phylogenetic tree was constructed using the neighbor-joining (NJ) method and plotted with MEGA 5.0 (<http://www.megasoftware.net/>).

Design and synthesis of primers

A pair of primers was designed by Primer 5.0 to amplify the *rpl40* gene of *T. gondii* (GenBank: XM_002368359.1). *Bam*HI and *Sal* I sites were introduced at the 5' ends of the forward and reverse primers, respectively. The forward primer 5'-GTTCG-GATCCATGCAGATTTTCGTG-3' and the reverse primer 5'-ACGGTCGACTTAGTTCTTGGGCTTC-3' were used to amplify a 930 bp fragment.

Parasite collection and RNA extraction

Toxoplasma gondii RH was recovered from liquid nitrogen. When each well of the tissue culture flask was 90% confluent with Vero cells, 1 × 10⁵ tachyzoites were inoculated into each well. Tachyzoites were collected by scraping and washing the flask with sterile saline three days post-inoculation. Tachyzoites were centrifuged at 1,000 rpm for 15 min and washed with phosphate buffered saline (PBS) three times prior to cell culture and RNA extraction. Total RNA was extracted by Trizol (Invitrogen, Carlsbad, CA, USA) and stored at -70°C until use.

PCR amplification and plasmid construction

cDNA was obtained from *T. gondii* RH total RNA by reverse transcription using a Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). Polymerase chain reaction (PCR) was performed using EX Taq DNA polymerase (Takara Bio Inc., Dalian, China) in a 50 µl reaction mixture containing 2 µl cDNA template. Reactions were performed for 30 cycles with denaturation at 95°C for 45 s, annealing at 52°C for 50 s, and extension at 72°C for 50 s. Products were analysed by electrophoresis in 1% agarose gels with Goldview (SBS Genetech Co., Guangzhou, China).

The purified PCR products were inserted into the pMD19T vector. Plasmids were then transformed into *Escherichia coli* TOP10 competent cells (CWBI Co., Beijing, China). Positive clones were screened on LB plates containing 100 µg/ml ampicillin. Gene sequencing was performed on positive clones by Invitrogen. The plasmid containing the PCR product was named pMD19T-TgRPL40. The plasmid was extracted using the Plasmid Miniprep Kit (Axygen Scientific Inc., Corning, NY, USA). Plasmid pMD19T-TgRPL40 was digested with *Bam*HI and *Sal* I and inserted into the *Bam*HI and *Sal* I sites of the pET-28a-c(+) expression vector. The sequence of the resulting plasmid was confirmed by dideoxy chain termination sequencing (Invitrogen).

Induction, expression, and purification of recombinant protein in *Escherichia coli*

The expression plasmid was transformed into *E. coli* BL21 competent cells (CWBI Co.). The transformed cells were grown in LB medium containing 70 µg/ml kanamycin at 37°C. Protein expression was induced with 0.5 mM isopropyl β-D-thiogalactopyranoside for 12 h at 20°C. Pelleted cells were re-suspended and disrupted with a Sonicator (Scientz Biotechnology Co., Ningbo, China). The cell lysate was centrifuged for 30 min at 12,000 × g and the precipitate was suspended in 25 ml binding buffer. After being filtered with a 0.22 µm membrane, the recombinant protein in the supernatant was purified by Ni-NTA His-bind Resin (Novagen, Billerica, MA, USA). The purified His-TgRPL40 was dialysed in PBS at 4°C for 12 h with stirring and then concentrated to 1 ml using a 3 kDa centrifugal filter (Merck, Darmstadt, Germany). The dialysed protein was stored at -80°C until use.

Antibody production

The concentration of the purified protein was measured using an RC DC Protein Assay kit (Bio-Rad, Hercules, CA, USA) after dialysis. Three mice were immunised with TgRPL40 protein absorbed to 100 µl 206 adjuvant (Seppic, Paris, France) at a dose of 100 µg per mouse. Immunisations were given at 0, 2, 4, and 6 weeks. Control group mice were injected with the same volume of PBS containing 206 adjuvant. Immunisation procedures were assessed and antibody titres were measured by enzyme-linked immunosorbent assay (ELISA). Blood samples were collected 10 days after each injection. Serum was separated by centrifugation and stored at -80 °C until use.

ELISA was performed to measure antibody concentrations in microliter polystyrene plates (flat bottom, low binding; Corning, NY, USA). The purified tachyzoites were lysed by ultrasonic. The supernatant was separated by centrifugation and filtered by 0.45 µm filter (Merck) to extract tachyzoite protein. A total of 500 ng tachyzoite protein dissolved in 100 µl coating buffer (pH 9.6) was added to each well. After incubating overnight at 4 °C, each well was blocked with 200 µl 1% gelatin at 37 °C for 3 h. Then, 100 µl diluted serum (1 : 4000) in 5% bovine serum PBST (phosphate buffered saline pH 7.2 containing 0.05% v/v Tween 20) was incubated at 37 °C for 1.5 h. After incubating with horseradish peroxidase-conjugated goat anti-mouse IgG (1 : 4000; Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA), 100 µl substrate solution was added to each well. The reaction was stopped by adding 50 µl 4 N H₂SO₄. The absorbance was measured at 450 nm using an ELISA reader (Thermo). Each serum sample was assayed in duplicate.

Western blotting

Sera from three immunised mice were collected and combined 3 days after intraperitoneal injection with *T. gondii* RH strain to analyse whether TgRPL40 present in CAg. About 2 ml of blood from each mouse was centrifuged at 2,000 rpm for 20 minutes to collect the serum. The sera were then filtered with 0.22 µm filter (Merck). ESA were prepared according to the method described previously (Xue et al. 2016). The purified tachyzoites were suspended in 15 ml serum-free DMEM medium and incubated in an incubator containing 5% CO₂ at 37 °C for 2 h. Then the medium was centrifuged at 4 °C for 15 min at 1,000 rpm. The supernatant was filtered by 0.45 µm filter (Merck) after adding protease inhibitor (Merck, Darmstadt, Germany).

The filtered supernatant was concentrated to about 1 ml through a protein concentrator with molecular weight of 3 kDa (Merck). The concentration of ESA was detected by DC protein assay reagent package (Bio-Rad, Hercules, USA). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with 100 µg of each sample loaded into each well. Protein bands on gels were transferred onto nitrocellulose membranes (0.45 µm; Amersham, Little Chalfont, UK) using a Trans-blot Semi-dry Transfer Cell (Bio-Rad). Mouse anti-TgRPL40 antiserum was used as the primary antibody (1 : 8000). Peroxidase-conjugated goat anti-mouse IgG (Jackson Immuno Research Laboratories, Inc.) was used as the secondary antibody. The membranes were visualised using a standard enhanced chemiluminescence system (Bio-Rad).

Immunofluorescence assay

Toxoplasma gondii-infected Vero cells grown on glass coverslips were fixed with 4% formaldehyde and permeabilised with 0.5% Triton-X100. The coverslips were blocked with PBS containing 1% bovine serum albumin (BSA) at 37 °C for 30 min. Mouse anti-TgRPL40 polyclonal antiserum (1 : 4000) was used as the primary antibody. Alexa Fluor 488-conjugated donkey anti-mouse IgG (Jackson Immuno Research Laboratories, Inc.) was used as the secondary antibody. Cells were stained with DAPI and images were acquired using Nikon Eclipse C1-Si confocal laser-scanning microscope (Nikon, Tokyo, Japan) with a 60× oil immersion lens. Three technical duplications were set for each sample.

Immunoelectron microscopy

For immunoelectron microscopy, extracellular tachyzoites were fixed with PBS containing 0.1% glutaraldehyde and embedded in LR white resin (Head Biotechnology Co., Beijing, China). Sections were treated with saturated sodium metaperiodate solution and incubated for 30 min in PBS containing 5% skim milk and 0.01% Tween 20 (PBS-milk-Tween). Grids were then placed in PBST containing 5% skim milk at 4 °C overnight. After washing with PBST containing 1% BSA (PBS-BSA-Tween), the sections were incubated for 1 h with mouse anti-TgRPL40 polyclonal antibody (diluted 1 : 100 in PBS-milk-Tween). After washing with PBS-BSA-Tween, grids were incubated for 1 h with gold particle-labeled goat anti-mouse IgG (1 : 20; 9–11 nm diameter; Cat No. SLBD7962V; Sigma-Aldrich Co., Santa Clara, CA, USA). The slides were then washed with PBS-BSA-Tween and distilled water. After staining with 50% methanol containing 2% uranyl acetate, the slides were observed using an electron microscope (Hitachi, Tokyo, Japan). Three technical duplications were set for each sample.

Evaluation of the protective effects of recombinant TgRPL40 in mice

To assess the immune protective effects of the recombinant TgRPL40 protein, mice were immunised with the protein at a dose of 100 µg per mouse as described above. An equal volume of PBS containing 206 adjuvant was injected into mice in the control group. Fourteen days after the third immunisation, both control and immunised mice (10 mice per group) were injected intraperitoneally with 4×10^5 parasites. Blank group mice were injected with the same volume of sterile PBS. Cumulative mortality was plotted using a Kaplan-Meier survival plot and analysed using Microsoft Excel 2011 (Microsoft, Redmond, WA, USA).

Analysis of tissue parasitism kinetics by quantitative real time PCR

To further determine the protective effect of the recombinant TgRPL40 protein, immunised and control mice (10 mice per group) were infected intraperitoneally with 4×10^4 *T. gondii* RH tachyzoites 14 days after the third immunisation. Two mice from each group were selected for collection of blood, liver, spleen, and brain on days 1, 3, 5, and 7 post-infection. To avoid sampling non-homogeneity in animals with low numbers of cysts, whole liver, spleen, or brain was ground in PBS. Blood, liver, spleen and brain tissue from the two mice from each group were combined.

Fig. 1. Phylogenetic tree analysis of TgRPL40 sequence. Multiple sequence alignment and phylogenetic analyses were conducted on the basis of observed similarities. Sequences were aligned with ClustalW2. The phylogenetic tree was constructed using the neighbor-joining (NJ) method and plotted using MEGA 5.0.

Liver (30 mg), spleen (10 mg) and brain (30 mg) tissue deposition was collected by centrifugation. DNA was extracted using a TIANamp Genomic DNA kit (Tiangen Biotech Co., Beijing, China).

Tissue parasitism kinetics was estimated by quantitative real time PCR (qPCR) using an ABI 7500 PCR system (Applied Biosystems, Foster City, CA, USA) (Lin et al. 2012). Purified DNA was used in the reaction after diluting samples to equal concentrations of DNA. Each sample was assessed in triplicate. Six 10-fold dilutions of *T. gondii* genomic DNA (0.1 ng to 1 fg) were used to calculate the standard curve. Each 25 µl reaction (0.4 µM primers, 0.3 µM probe and 1 µl template) used the Probe qPCR Master Mix with uracil-DNA-glycosylase (Thermo Fisher Scientific). After 2 min at 50°C and an initial denaturation at 95°C for 10 min, amplification consisted of 40 cycles of denaturation at 95°C for 15 s, annealing at 52°C for 10 s and extension at 72°C for 15 s. Parasite loads are presented as the numbers of tachyzoites in each g of tissue or per ml of blood.

Statistical analysis

Statistical analysis was performed using SPSS version 17.0 for Windows (SPSS Inc., Chicago, IL, USA). Data were analysed using one-way analysis of variance (ANOVA) followed by the least significant difference test. Data are expressed as mean ± standard deviation. Differences were considered to be statistically significant at $P < 0.05$ or $P < 0.01$.

RESULTS

Phylogenetic tree analysis indicates that TgRPL40 is highly conserved in different species

NCBI online tools and DNASTar software were used to analyse the amino acid sequence of the TgRPL40 protein. BLAST results showed that the RPL40 protein is highly

conserved in protists. The sequence of *Toxoplasma gondii* RH TgRPL40 is highly homologous to the protein sequences of RPL40 proteins from a variety of other *T. gondii* strains (identity > 95%). Phylogenetic tree analysis showed that the sequence of the RPL40 protein of *T. gondii* RH, ME49, and VEG strains is highly homologous to the RPL40 protein of other protists, such as *Hammondia hammondi* Frenkel et Dubey, 1975 and *Neospora caninum* Dubey, Carpenter, Speer, Topper et Ugglä, 1988 (Fig. 1) from Liverpool (identity > 95%).

Compared with other species, differences in amino acid residues were found mainly at the N terminus. TgRPL40 contains 129 amino acid residues and amino acids 125–129 are -KKPKN. The amino acid sequences of the RPL40 protein of parasitic protists, such as *H. hammondi* and *N. caninum* (strain Liverpool), are the same as that of TgRPL40. In addition to parasitic protists, the RPL40 protein of *Drosophila ficusphila* Kikkawa et Peng, 1938 has the highest homology with TgRPL40. The RPL40 of *D. ficusphila* contains 128 amino acid residues and amino acids 125–128 are -KKLK.

Recombinant protein can be highly expressed by *E. coli* and is immunogenic

Secondary structure analysis revealed high peptide epitope prediction scores for the peptide encoded by the 930 bp fragment. SDS-PAGE results showed that the recombinant TgRPL40 protein was expressed. The recombinant protein fused with a His-tag was 18.25 kDa and could be purified by Ni-NTA resin (Fig. 2A). The antibody titres of the mice immunised with the recombinant protein were 1 : 4000 after the fourth immunisation. High titres indicated that the recombinant TgRPL40 protein was immunogenic (Fig. 2D).

Fig. 2. Protein expression, Western blotting and antibody preparation. **A** – The recombinant TgRPL40 protein was expressed in an *Escherichia coli* expression system and purified using Ni-NTA resin. The purified protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 18%) and the gel was stained with Coomassie brilliant blue-R250; **B** – TgRPL40 in the serum of mice acutely infected with *Toxoplasma gondii* (Nicolle et Manceaux, 1908) was identified with recombinant TgRPL40 antiserum. Control serum was obtained from three healthy mice; **C** – TgRPL40 in excretory/secretory antigens (ESA) was identified by recombinant TgRPL40 antiserum; **D** – Antibody titres were detected by enzyme-linked immunosorbent assay (ELISA). The interval between immunisations was two weeks. Sera were collected from the tails of mice approximately 2 weeks after each immunisation.

Fig. 3. Subcellular localisation using immunofluorescence and immunoelectron microscopy. **A** – subcellular localisation was performed using immunofluorescence; **B** – controls used in immunoelectron microscopy; **C** – Distribution of TgRPL40 in a longitudinal section of a tachyzoite; **D** – the enlargement of the local visual field in panel C.

TgRPL40 is a component of CAG and ESA

The present study showed that TgRPL40 could be detected by immunoblotting in sera of mice acutely infected with *T. gondii* (Fig. 2B). In addition, we identified TgRPL40 in the ESA (Fig. 2C). These results indicate that TgRPL40 is a CAG component, which is consistent with our

previous proteomic study of acute infection with *T. gondii* infection Xue et al. (2016).

TgRPL40 is widely distributed in the tachyzoites

Indirect immunofluorescence assays showed that TgRPL40 was localised mainly in the cytoplasm of tachyzoites (Fig. 3A). To further investigate the potential function of

Fig. 4. Dynamic changes of *Toxoplasma gondii* (Nicolle et Manceaux, 1908) in blood, liver, spleen, and brain of mice. **A–D** – Ten mice in the protection group were immunised with recombinant TgRPL40 protein. Control group mice were immunised with the same volume of PBS. Each mouse was injected intraperitoneally with 4×10^4 parasites 14 days after the third immunisation. The numbers and dynamic changes of *T. gondii* in different tissues were detected by qPCR on days 1, 3, 5, and 7 post-infection. Values represent mean $\pm$ standard deviation. Groups were compared using one-way analysis of variance (ANOVA) followed by a least significant difference test. Significant differences relative to controls are indicated by * ($P < 0.05$) or ** ($P < 0.01$).

TgRPL40, the intracellular location of the protein was determined using a more sensitive immunoelectron microscopy assay. The results of this assay confirmed that the TgRPL40 protein was widely distributed in the cytoplasm of tachyzoites. This distribution is consistent with the results of indirect immunofluorescence tests (Fig. 3B–D).

Immunisation with recombinant TgRPL40 protein reduces tachyzoite burdens in blood, liver, spleen, and brain

To monitor parasitemia and further assess the protective effects of recombinant TgRPL40 in mice, tissue parasitism kinetics were analysed by qPCR. The amplification efficiency for each test of *T. gondii* numbers in different tissues is shown in Supplementary Tab. 1. Tachyzoite numbers in the blood and livers of mice in the immunised group were significantly lower 7 days after infection (ANOVA test, $P < 0.01$) (Fig. 4A,B). Tachyzoite numbers were also significantly lower in spleen and brain of mice in the immunised group 7 days after infection (ANOVA test, $P < 0.05$) (Fig. 4C and 4D). *Toxoplasma gondii* became detectable by qPCR in brain tissue later than in the other tissues. These data suggest that immunisation with recombinant TgR-

PL40 exerts different degrees of inhibition on tachyzoites in blood, liver, spleen, and brain.

Immunisation with recombinant TgRPL40 prolongs survival of mice infected with *T. gondii*

To evaluate the protective effect of immunisation with recombinant TgRPL40 in mice, tachyzoites (4×10^5) were intraperitoneally injected into ICR mice (~8 weeks old) 14 days after the third immunisation. Mice immunised with recombinant TgRPL40 survived longer than mice that were immunised with PBS. However, mice immunised with the recombinant protein still eventually succumbed to the infection. All mice immunised with PBS died within 8 days, whereas mice immunised with recombinant TgR-PL40 survived for 9 to 17 days (Fig. 5).

DISCUSSION

Our previous results indicated that TgRPL40 may play an essential role in the pathogenesis of acute infection with *Toxoplasma gondii* (see Xue et al. 2016). TgRPL40 is a fusion of ubiquitin and ribosomal protein L40. In most eukaryotic cells, ubiquitin is composed of 76 amino acid residues (Catic and Ploegh 2005). There are three precursor forms of ubiquitin including polyubiquitin and two N-ter-

Fig. 5. Survival curves of mice infected with *Toxoplasma gondii* (Nicolle et Manceaux, 1908). Mice were immunised with recombinant TgRPL40. Control mice were administered phosphate buffered saline (PBS). Fourteen days after the third immunisation mice were injected intraperitoneally with 4×10^5 parasites. Blank group mice were injected with the same volume of PBS. Survival was monitored for 20 days.

minimal ubiquitin moieties. The moieties Ubl40 and UbS27 are fused with the ribosomal polypeptides L40 and S27, respectively. Cleavage of these precursor molecules by specific endopeptidases releases the ubiquitin monomers. The ubiquitin moiety then acts as a molecular chaperone to promote ribosome assembly before cleavage by L40 or S37 (Catic and Ploegh 2005).

Our results indicate that the pattern of TgRPL40 is similar to that of RPL40 in other species, which play roles in cytoplasm and some organelles (Wang et al. 2012, Fernandez-Pevida et al. 2012). The wide distribution of TgRPL40 in tachyzoites may be related to its physiological function. In other species, ubiquitin has been shown to have multiple roles during biological processes, including apoptosis, transcription and cell cycle, through covalent interactions with other proteins (Chi et al. 2016). First, it is well known that ubiquitylation is a biological signal of protein degradation. The isopeptide linkages between the glycine (G) 76 and lysine (K) 48 or K29 of ubiquitin initiates enzyme-dependent degradation of ubiquitin binding proteins (Pickart 2000, 2001). Second, recent studies also indicate that ubiquitin plays an essential role in intracellular signal transduction (Su et al. 2013, Dores and Trejo 2019). Other biological processes include the regulation of ribosomal function (Spence et al. 2000), transcription (Bhat and Greer 2011), mitochondrial DNA inheritance (Lee et al. 2010) and DNA repair (Hedglin and Benkovic 2015).

Ribosomal proteins are also structural components of the ribosome and play a role in protein translation and processing of genetic information (Han et al. 2012). Ribosomal proteins usually surround rRNA cores of ribosomal subunits. Although the amino acids encoding the peptides

are based on RNA, ribosomal proteins may play roles in simplifying the protein translation process and other biological processes outside of the ribosome (Lee et al. 2013, Gämderinger 2016). In this study, subcellular localisation indicated that the ribosomal TgRPL40 fusion protein has functions in the cytoplasm of tachyzoites.

The TgRPL40 antigen was identified in ESA of *T. gondii* by immunoblotting. TgRPL40 has also been identified as a CAg through proteomic analysis of serum collected during acute toxoplasmosis (Xue et al. 2016). Because signal peptide prediction analysis showed that there is no signal peptide in TgRPL40, we speculate that this protein may be secreted via exosomes or by other mechanisms. Although previous studies have focused on the functions of RPL40, the function of TgRPL40 in *T. gondii* infection and other biological processes was not sufficiently analysed. Expression of RPL40 in HEK293 cells is required for the cells to transition from an adherent to a suspension phenotype after heat shock (Trakman et al. 2016).

RPL40 plays a role in the formation of vesicular stomatitis virus 80S mRNA through a cis-regulatory element (Lee et al. 2013). In the current study, we observed a high degree of homology with other parasitic protists, such as *Hammondia hammondi* and *Neospora caninum*, suggesting that TgRPL40 may have similar functions in counteracting host immune mechanisms. Similarly, human IgM and IgG antibodies have been shown to demonstrate high sensitivity and specificity for ribosomal ubiquitin proteins and the polyubiquitin of *T. gondii* Saadatnia et al. (2011). Why immune recombinant TgRPL40 protein can cause immune protection and the mechanism of its antibodies to play an immune protective role remain to be further studied.

Our study demonstrates that *T. gondii* ubiquitin can be expressed in the form of a fusion protein with ribosomal subunit L40 in tachyzoites. Immunisation with recombinant TgRPL40 can prolong the survival time of mice infected with *T. gondii*. We also demonstrated that recombinant TgRPL40 reduces the tachyzoite burden in blood, liver, spleen and brain of mice infected with *T. gondii*. These outcomes show that TgRPL40 is a potential candidate molecule for the prevention of *T. gondii*. Although immunisation with TgRPL40 alone was not able to prevent the death of mice infected with *T. gondii*, further study of the protective effects of TgRPL40 combined with other virulence factors will identify optimal combinations of virulence factors for the development of vaccines for clinical application.

Acknowledgments. This work was supported by National Key Research and Development Program of China (Grant No. 2016YFD0501100, 2016YFC1202000, 2016YFC1202004) and Shanghai Natural Science Foundation of China (Grant No. 17ZR1437300, 18ZR1413400).

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Received 7 December 2018

Accepted 22 May 2019

Published online 27 September 2019

Cite this article as: Xue J., Jiang W., Li J., Xiong W., Tian Z., Zhang Q., Li S., Liu C., Huang K., Wang Q. 2019: *Toxoplasma gondii* RPL40 is a circulating antigen with immune protection effect. *Folia Parasitol.* 66: 013.